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Effect of Hypoglycemia on Inflammatory Responses and the Response to Low Dose Endotoxemia in Humans

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Abbreviations: ACCORD, Action to Control Cardiovascular Risk in Diabetes; AMI, acute myocardial infarction; APC, allophycocyanin; AR1, autoregressive correlation structure; AUC, area under the curve; BMI, body mass index; CCL2, C-C motif chemokine ligand 2; CM, classical monocytes; CM-MPA, classical monocyte-platelet aggregates; CV, cardiovascular; CXCL8, C-X-C motif chemokine ligand 8; CX_3CL1, CX_3C chemokine ligand 1; CX_3CR1, CX_3C chemokine receptor 1; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; IM, intermediate monocytes; IM-MPA. intermediate monocyte-platelet aggregates; MPA, monocyte-platelet aggregates; NaCl, sodium chloride; NCM, non-

classical monocytes; NCM-MPA, non-classical monocyte-platelet aggregates; PBS, phosphate-buffered saline; PE, phycoerythrin; STEMI, ST-elevation myocardial infarction; WBC, white blood cell.

Précis: Using a novel *in vivo* human experimental model, we show that hypoglycemia primes the innate immune system leading to a more profound inflammatory response to a subsequent inflammatory stimulus.

Abstract

Context

Hypoglycemia is emerging as a risk for cardiovascular events in diabetes. We hypothesized that hypoglycemia activates the innate immune system, which is known to increase cardiovascular risk.

Objective

To determine whether hypoglycemia modifies subsequent innate immune system responses.

Design and Setting

Single-blinded, prospective study of three independent parallel groups.

Participants and Interventions

Twenty-four healthy participants underwent either a hyperinsulinemic-hypoglycemic (2.5 mmol/l), euglycemic (6.0 mmol/l) or sham-saline clamp (n=8 for each group). Forty-eight hours later, all participants received low-dose (0.3 ng/kg) intravenous endotoxin.

Main outcome measures

We studied *in-vivo* monocyte mobilization and monocyte-platelet interactions.

Results

Hypoglycemia increased total leucocytes (9.98±1.14 x10^9/l vs euglycemia: 4.38±0.53 x10^9/l; P<0.001 vs sham-saline: 4.76±0.36 x10^9/l; P<0.001) (mean±SEM), mobilized proinflammatory intermediate monocytes (42.20±7.52/µl vs euglycemia: 20.66±3.43/µl; P<0.01 vs sham-saline: 26.20±3.86/µl; P<0.05) and non-classical monocytes (36.16±4.66/µl vs euglycemia: 12.72±2.42/µl; P<0.001 vs sham-saline: 19.05±3.81/µl; P<0.001). Following hypoglycemia vs euglycemia, platelet aggregation to agonist (AUC) increased (73.87±7.30 vs 52.50±4.04; P<0.05) and formation of monocyte-platelet aggregates increased (96.05±14.51/µl vs 49.32±6.41/µl; P<0.05). Within monocyte subsets, hypoglycemia increased aggregation of intermediate monocytes (10.51±1.42/µl vs euglycemia: 4.19±1.08/µl; P<0.05 vs sham-saline: 3.81±1.42/µl; P<0.05) and non-classical monocytes (9.53±1.08/µl vs euglycemia: 2.86±0.72/µl; P<0.01 vs sham-saline: 3.08±1.01/µl; P<0.05) with platelets compared to controls. Hypoglycemia led to greater leucocyte mobilization in response to subsequent low-dose endotoxin challenge (10.96±0.97 vs euglycemia: 8.21±0.85 x10^9/l; P<0.05).

Conclusions

Hypoglycemia mobilizes monocytes, increases platelet reactivity, promotes interaction between platelets and proinflammatory monocytes, and potentiates the subsequent immune response to endotoxin. These changes may contribute towards increased cardiovascular risk observed in people with diabetes.

Introduction

Hypoglycemia is associated with a greater propensity to adverse cardiovascular (CV) outcomes in diabetes (1-3). To determine if such outcomes were dependent upon changes in innate immune responses, we devised a novel model whereby subjects were challenged with a hypoglycemic clamp, and then the durable effects on the innate immune system probed by an *in vivo* endotoxin challenge 48 hours later.

Iatrogenic hypoglycemia remains a major barrier to effective treatment of insulin-treated diabetes (4). The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial showed that intensive glucose control, during which patients were exposed to significantly more hypoglycemia (5), was associated with excess CV mortality. Despite the evidence confirming an association between hypoglycemia and mortality, cause and effect has not been established. Trial evidence suggests that the relationship is, at least in part, explained by 'confounding', i.e. that hypoglycemia identifies individuals with comorbidities who are both vulnerable to hypoglycemia and more likely to die for other reasons (6). Nevertheless, a recent large meta-analysis (7) has suggested that comorbidities alone are unlikely to explain this relationship. Furthermore, there is a growing body of evidence highlighting a number of mechanisms whereby hypoglycemia may lead to CV events (8, 9).

Hypoglycemia has proinflammatory consequences, including increases in levels of factor VIII and von Willebrand factor and impaired fibrinolysis (10-12). In addition, hypoglycemia has been shown to increase proinflammatory cytokines (12-14) and promote rises in the levels of proatherogenic cell adhesion molecules (12). Repeated episodes of hypoglycemia have also been reported to impair nitric oxide-mediated vasodilation (15).

Monocytes are phagocytes that are central to the etiology of atherosclerosis (16) and play a role in precipitating acute CV events by promoting plaque destabilization and rupture (17). The extent to which monocytosis and monocyte activation is modified by hypoglycemia remains uncertain. Recent studies have also determined that monocytes can be classified into 3 distinct subsets, termed classical monocytes (CM: $CD14^{++}$ $CD16^{-}$, 'Mon1'), intermediate monocytes (IM: $CD14^{++}$ $CD16^{+}$, 'Mon2'), and non-classical monocytes (NCM: $CD14^{+}$ $CD16^{++}$, 'Mon3') (18, 19). A number of observational studies indicate that IM may be particularly proatherogenic. Elevated levels of IM are associated with adverse CV outcomes (20-23), independently predict future CV events (22), and have been associated with coronary plaque vulnerability in patients with angina (24). Elevated levels of CM may also independently predict CV events (25).

Acute myocardial infarction (AMI) results in monocytosis, mediated by sympathetic nervous system activation (26). In humans, $CD16^{+}$ monocytes selectively mobilize, in a catecholamine-dependent fashion, following exercise (27). As epinephrine is the key counter-regulatory hormone produced in response to hypoglycemia, we hypothesized that hypoglycemia would also exert significant effects on monocytes. We further hypothesized that we would see additional synergistic changes in monocyte and platelet activation, as revealed by formation of monocyte-platelet aggregates, which are increased after AMI (20, 28). In large prospective studies, CV events did not appear to occur during the hypoglycemic episode *per se* but there was an increased risk of events in the weeks and months following the episode (29-31). We hypothesized, therefore, that acute hypoglycemia may prime the innate immune system, leading to a more pronounced inflammatory response to a subsequent inflammatory stimulus downstream from the initial episode of hypoglycemia. It is also relevant to note that people with diabetes experience increased incidences of acute and

chronic infections that will further activate innate immunity. To reveal if hypoglycemia modulated monocyte function in the human *in vivo*, we chose to combine a classical hypoglycemic stimulus with a subsequent *in vivo* systemic stimulus of the innate immune system. To achieve this, we combined hyperinsulinemic hypoglycemic-euglycemic and sham-saline clamps with low-dose intravenous endotoxin challenge 48 hours later in healthy participants. Endotoxin, otherwise known as Gram-negative bacterial lipopolysaccharide, was employed as it induces a short-lived, sterile inflammation that is both safe and reproducible (32).

Materials and Methods

Study Design and Participants

This was a single-blinded, prospective study of three independent parallel groups (hyperinsulinemic-hypoglycemia/euglycemia and sham-saline controls) conducted in a random group order at the Clinical Research Facility, Northern General Hospital, Sheffield, United Kingdom between January 2015 and April 2016. We therefore had three groups that had euglycemia with insulin, hypoglycaemia with insulin, or saline. Each then received endotoxin. Baseline values at the start of endotoxin administration were studied in all groups, providing a set of data obtained prior to endotoxin. Twenty-four healthy participants without diabetes were recruited from the University of Sheffield and Sheffield Teaching Hospitals with written informed consent in accordance with a protocol approved by Yorkshire and the Humber-Sheffield Research Ethics Committee (REC 14/YH/1264). All participants had a HbA_{1C} < 6.5% (< 48 mmol/mol), measured using ion-exchange high-performance liquid chromatography, and none had impaired glucose tolerance based on HbA_{1C} as judged by the American Diabetes Association criteria (33, 34). Participants were in good health as determined by a medical history, physical examination, vital signs and clinical laboratory test

results including full blood count and renal and liver function. Those with an intercurrent illness in the previous 4 weeks were excluded. Participants taking beta-blockers, QT interval-prolonging agents and anticoagulant, antiplatelet or antiinflammatory medications were also excluded. Female participants were on secure contraception and also had negative urinary pregnancy tests on the morning of the clamp and endotoxin studies.

Clamp studies

All participants attended at 0800h after an overnight fast and were blinded to their group allocation. Participants were instructed to avoid caffeine, alcohol and vigorous exercise 24 hours before the study visit. An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm for insulin and dextrose infusion. A second intravenous cannula was inserted into the antecubital fossa of the dominant arm for all blood measurements except glucose. Following the application of a local anesthetic cream (EMLA, Astra-Zeneca, Macclesfield, UK) to the dorsal hand or wrist of the non-dominant arm, a retrograde cannula was inserted and the hand placed in a warming chamber (The Sheffield Hand Warmer, Sheffield, UK) at 55^{o}C to allow arterialisation of venous blood for glucose measurement. In hypoglycemia and euglycemia study groups, a primed continuous insulin (Human Actrapid, Novo Nordisk Pharmaceuticals LT, Crawley, UK) infusion was administered at a rate of 90 mU m^{-2} min^{-1} with total insulin exposure matched between groups. A 20% dextrose (Baxter, Baxter Healthcare Ltd., Thetford, UK) variable rate infusion was administered simultaneously and the rate adjusted according to arterialized whole blood glucose concentrations measured every 5 minutes using a glucose oxidase method (Yellow Springs Instrument 2300 STAT, Yellow Springs, Ohio, USA). Following a brief (30 minutes) euglycemic phase in both groups, blood glucose was lowered to 2.5 mmol/l in the hypoglycemia group and maintained for 60 minutes at this level. In the euglycemia group,

blood glucose was maintained at 6 mmol/l for 60 minutes. Participants in the sham-saline group were investigated identically but did not receive insulin/dextrose infusions and instead received a slow intravenous infusion of 0.9% sodium chloride (NaCl) (Baxter, Baxter Healthcare Ltd., Thetford, UK) at a pre-determined fixed rate. Thus, participants in the sham-saline group were under normoglycemic conditions allowing us to control for the effects of insulin and dextrose. Blood was sampled at baseline and at 60 minutes. Members of staff processing assays were blinded to glucose group allocation.

Endotoxin challenge

Endotoxin challenge is a safe and well-studied model of innate immune activation *in vivo* (35). Forty-eight hours following the clamp, participants re-attended at 0800h having fasted overnight and refrained from caffeine, alcohol and vigorous exercise since the clamp visit. An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm for administration of endotoxin and a second cannula inserted into the contralateral antecubital fossa for blood sampling. All participants received 0.3 ng/kg *Escherichia coli* O:113 lipopolysaccharide (Clinical Centre Reference Endotoxin, National Institutes of Health, Bethesda, Maryland, USA). Endotoxin powder was reconstituted in 1 ml of sterile 0.9% NaCl to form a solution at a concentration of 1000 ng/ml, which was vortexed for 60 minutes. The weight-adjusted dose of endotoxin was obtained from this solution, added to 5 ml of 0.9% NaCl and administered as a slow bolus injection over 1 minute. An intravenous infusion of 500 ml of 0.9% NaCl (Baxter, Baxter Healthcare Ltd., Thetford, Norfolk, UK) then continued for 4 hours following endotoxin to avoid hypotension. Venous blood was sampled at baseline and 2, 4 and 6 hours following endotoxin. All laboratory measurements were performed by staff blinded to glucose group allocation.

Biochemical analysis

To measure epinephrine, venous forearm blood was collected into chilled lithium heparin tubes and centrifuged at 4ºC, 1000*g* for 10 minutes. The resulting supernatant was stored at -80ºC until assayed by high-performance liquid chromatography. To determine insulin levels, EDTA-anticoagulated blood was centrifuged at 3000*g* for 10 minutes, and free insulin levels measured in the resulting plasma using an immunoassay (Roche Cobas, Roche Diagnostics, Burgess Hill, West Sussex, UK). Venous blood was centrifuged at 3000*g* for 10 minutes and the resulting serum used to measure cortisol and growth hormone using an immunoradiometric assay (Roche Cobas, Roche Diagnostics, Burgess Hill, West Sussex, UK). Sample collection for cortisol and growth hormone was controlled for time of day across the three study groups.

Cell counts and flow cytometry

Total and differential white blood cell (WBC) and platelet counts in EDTA-anticoagulated blood were determined using an automated clinical grade Sysmex cell counter (XN-9000, Sysmex, Milton Keynes, UK). For the clamp visit, alternative WBC counting methodologies were piloted for the first two subjects in each group but these were later deemed less accurate than the Sysmex cell counter. Data shown below in Figures 2, 3 and 4a, 4c, 4d, 4e and 4f are Sysmex data from n=6 in each study group. Flow cytometry was used to determine monocyte-platelet aggregates (MPA): blood was collected into tubes containing trisodium citrate dihydrate (3.13% w/v) and incubated in a heat block at 37ºC for 10 minutes, erythrocytes lysed using fluorescence-activated cell sorting (FACS) lyse solution (BD, Oxford, UK) and stained with FITC-conjugated CD16 (BioLegend, London, UK), APC-conjugated CD14 (BioLegend, London, UK) and PE-conjugated CD42a (BD, Oxford, UK) in

addition to matched isotype controls. Cells were fixed using FACS Fix (BD, Oxford, UK) and analyzed using flow cytometry (Accuri C6 multi-colour flow cytometer, BD, Oxford, UK) within a consistent time frame for all subjects. Monocytes were gated based on morphology and CD14 expression. Neutrophils were gated on morphology and through exclusion of monocytes. Monocyte-platelet aggregation was determined by measuring monocyte mean fluorescence of the platelet-specific marker CD42a. To phenotype and enumerate monocyte subsets, anticoagulated blood was stained with FITC-conjugated CD16 (BioLegend, London, UK), APC-conjugated CD14 (BioLegend, London, UK), PE-conjugated CD66c (BD Biosciences, Oxford, UK), PE-Cy7-conjugated CD11b (BioLegend, London, UK) and (PerCP)-eFluor® 710-conjugated CX3CR1 (eBioscience, Altrincham, UK). Matched isotype controls and a 'fluorescence minus one' strategy optimized compensation. Stained whole blood was lysed with FACS lyse as above, and the pellet resuspended in PBS prior to fixation using 1% w/v formaldehyde. Samples were immediately processed for analysis using flow cytometry (LSRII, BD, Oxford, UK). Monocytes were gated based on morphological characteristics and through the exclusion of neutrophils using CD66c. Monocyte subsets were identified based on relative expression of CD14 and CD16. Flow cytometry data were analysed using Flow Jo (FlowJo, version 10, Ashland, Oregon, USA).

Platelet aggregation

Platelet aggregation was measured using impedance aggregometry (Multiplate®, Verum Diagnostica GmBH, Munich, Germany). Aliquots of 300µl saline and 300µl hirudin-anticoagulated blood were added to the cuvette and incubated at 37 ºC for 3 minutes. Twenty microliters of adenosine diphosphate (ADP, at a final concentration of 6.45µM) was added as

agonist and the assay commenced. The area-under-the curve (AUC) was measured, which represents the level of platelet aggregation.

Statistical analysis

Our pilot data indicated that a sample size of 7 participants per group would have 90% power to detect a 50% relative difference in mobilization of monocytes between hypoglycemia and controls. Eight subjects were recruited per group to allow for a 13% drop out rate. Mean baseline measurements of glucose were compared between groups using analysis of variance (ANOVA). Mean measurements of glucose, insulin, and epinephrine at 60 minutes post clamp were compared, adjusting for clamp baseline measurement, using analysis of covariance (ANCOVA). In the event of unequal variance between groups, a log transform was applied and the analysis repeated. Longitudinal and between-group comparisons were made for post-endotoxin measurements using mixed effects linear models. For models examining between-group differences, the baseline endotoxin measurement was included as a covariate. For all mixed-effects linear models, an autoregressive correlation structure (AR1) was used to allow for the correlation between multiple measurements on the same person. Planned contrasts were made versus baseline and between groups at equivalent time points with Sidak's correction for multiple comparisons. All data are expressed as mean ± SEM, unless otherwise specified, and a P value of < 0.05 was deemed statistically significant. Analysis was performed using SPSS (version 22.0, IBM, Chicago, Illinois, USA).

Results

Participants

Study participants across the three groups were well matched for age, sex, BMI, HbA_{1C} and total WBC count with no significant differences at screening (Table 1). Participant numbers at each stage of study is illustrated in a flow diagram (Supplemental flow diagram).

Clamp studies

Glucose, insulin and counter regulatory hormones

Arterialized blood glucose values are shown in Fig. 1a. The glucose values were 2.51 ± 0.11 mmol/l and 6.04 ± 0.16 mmol/l at the end of the hypoglycemia and euglycemia clamps, respectively. Glucose values at the end of the sham-saline clamp were 4.64 ± 0.09 mmol/l. A counterregulatory response to hypoglycemia was evident with epinephrine levels during hypoglycemia (1.87 ± 0.25 nmol/l) being significantly higher ($P < 0.001$) compared to euglycemia (0.07 ± 0.01 nmol/l) and sham-saline (0.10 ± 0.04 nmol/l) (Fig. 1b). Free insulin levels at the end of clamp were similar between hypoglycemia (968.5 ± 149.1 pmol/l) and euglycemia groups (1025.4 ± 81.4 pmol/l, $P = 0.996$) but significantly higher ($P < 0.001$) then those in the sham-saline (31.3 ± 6.3 pmol/l) group (Fig. 1c). Serum cortisol and growth hormone were significantly higher in the hypoglycemia group compared to euglycemia and sham-saline controls (Fig. 1d and 1e).

Total and differential leucocyte count

We determined if hypoglycemia results in changes in circulating leucocytes. Hypoglycemia significantly increased the total number of WBC compared to controls (Fig. 2a). There was an increase across all classes of leucocytes studied, including neutrophils (Fig. 2b), lymphocytes (Fig. 2c) and total monocytes (Fig. 3a).

Monocyte subsets

We sought to determine if hypoglycemia exerted specific effects on monocyte subsets associated with cardiac pathology. Hypoglycemia increased the absolute number of all three circulating monocyte subsets compared to euglycemia and sham-saline (Fig. 3b-d). The

number of circulating NCM after 60 minutes of hypoglycemia compared to baseline (17.6 ± 2.9 cells/μl) increased twofold. IM numbers after 60 minutes of hypoglycemia compared to baseline (23.2 ± 4.5 cells/μl) increased by a factor of 1.81 and CM after 60 minutes of hypoglycemia compared to baseline (442.4 ± 55.3 cells/μl) increased by a factor of 1.29. There were no significant differences in the baseline values of all three monocyte subsets between the study groups.

Platelet count, aggregation and monocyte-platelet aggregates

Activation of platelets and generation of platelet-leucocyte aggregates contribute to leucocyte mobilization and inflammation in the vasculature (36). We therefore studied platelet number and function and their interaction with leucocytes. Total platelet count increased in hypoglycemia compared to euglycemia and sham-saline controls (Fig. 4a). ADP-induced platelet aggregation increased following 60 minutes of hypoglycemia versus euglycemia (P = 0.014) and there was numerically, but not statistically significantly, higher platelet aggregation detected in the hypoglycemia group compared to sham-saline group (P = 0.064) (Fig. 4b). The total number of MPA increased following 60 minutes of hypoglycemia compared to euglycemia (Fig. 4c). Whilst total MPA were not significantly higher in hypoglycemia compared to sham-saline controls at 60 minutes (Fig. 4c), we observed specific increases in non-classical monocyte (NCM) and intermediate monocyte (IM)-platelet aggregates (Fig. 4d and 4e). Classical monocyte (CM)-platelet aggregates appeared to increase following 60 minutes of hypoglycemia versus euglycemia and sham-saline but this was not statistically significant (P = 0.054) (Fig. 4f).

Cell surface markers

To further explore the activation state of monocytes after hypoglycemia, we studied expression levels of chemokine receptor CX_3CR1 and integrin CD11b. Hypoglycemia did not alter the expression of CX_3CR1 or CD11b (Supplemental Fig. 1a and 1b).

Endotoxin challenge

To determine if prior hypoglycemia affected the subsequent response to a classical immune activator, and thus to reveal if hypoglycemia had any longer-lasting effects on the innate immune system, we next proceeded to a low-dose intravenous endotoxin challenge 48 hours after the hypoglycemic challenge in all subjects. Consistent with the low-dose model employed, no fever or significant change in mean arterial blood pressure was recorded following endotoxin challenge across the study groups.

Epinephrine, cortisol and growth hormone

In contrast to the stress response induced by hypoglycemia, epinephrine levels were not significantly different between study groups 6 hours following endotoxin administration (Fig. 5a). In the hypoglycemia group, epinephrine levels were 0.15 ± 0.04 nmol/l versus 0.06 ± 0.01 nmol/l in euglycemia group and 0.09 ± 0.01 nmol/l in sham-saline group. There were also no differences detected between groups in serum cortisol and growth hormone levels following endotoxin administration (Fig. 5b and 5c). However, a rise versus baseline in the stress hormone cortisol was evident whereby serum cortisol levels peaked at 4 hours following endotoxin challenge in all study groups ($P = 0.005$) (Fig. 5b).

369

370 **Total and differential leucocyte count**

371 We observed that antecedent hypoglycemia modulated the subsequent WBC response to
372 endotoxin. Total number of WBC increased significantly following endotoxin in all the study
373 groups (Fig. 6a). The peak WBC response occurred at 4 hours post endotoxin and this was
374 significantly higher in the hypoglycemia group at $10.96 \pm 0.97 \times 10^9$/l vs $8.21 \pm 0.85 \times 10^9$/l in
375 the euglycemia group ($P = 0.012$) (Fig. 6a). Total WBC count 4 hours post endotoxin in the
376 sham-saline group was $10.65 \pm 0.64 \times 10^9$/l and this was significantly higher compared to
377 euglycemia ($P = 0.033$), but not hypoglycemia ($P = 0.974$). The rise in WBC was mainly a
378 consequence of an increase in neutrophil count (Fig. 6b). The lymphocyte count decreased
379 following endotoxin (Fig. 6c) and the monocyte count initially decreased prior to recovery 6
380 hours post endotoxin (Fig. 7a). There was a trend towards a higher total monocyte count in
381 the hypoglycemia group 4 hours post endotoxin compared to euglycemia but this comparison
382 did not reach statistical significance ($P = 0.085$). The absolute number of circulating
383 monocyte subsets did not differ significantly between study groups (Fig. 7b, 7c and 7d).
384 NCM and IM numbers decreased significantly following endotoxin compared to baseline
385 values in all groups ($P < 0.001$) (Fig. 7b and 7c). Compared to baseline, CM numbers
386 significantly declined at 2 hours ($P < 0.001$), prior to rising and reaching a peak at 6 hours (P
387 < 0.001) (Fig. 7d).

388

389 **Cell surface markers**

390 We examined monocyte activation following endotoxin exposure by measurement of cell
391 surface marker CX_3CR1 expression. Endotoxin administration caused a significant decline in
392 expression of this marker across all study groups compared to baseline ($P < 0.001$)
393 (Supplemental Fig. 2a). This was accompanied by an increase in the concentration of

CX_3CL1 in plasma at 4 and 6 hours compared to baseline in all groups ($P < 0.001$) (Supplemental Fig. 2e). Activation of monocytes was also revealed by increased expression of CD11b expression at 4 and 6 hours following endotoxin compared to baseline in all groups ($P < 0.001$) (Supplemental Fig. 3a). In addition, the percentage of total monocytes that were positive for CD11b expression was higher in hypoglycemia group versus euglycemia group at 2 hours post endotoxin ($P = 0.007$) (Supplemental Fig. 3b).

Discussion

Hypoglycemia may contribute to exacerbations of ischemic CV disease. We aimed to investigate the effect of acute experimental hypoglycemia and subsequent low-dose endotoxemia on aspects of the innate immune response (total leucocytes, leucocyte subsets and specifically monocyte subsets), thrombosis (platelet aggregation) and cross-talk between inflammation and thrombosis (monocyte-platelet aggregates). Our main findings were: (1) hypoglycemia increased the number of all three circulating monocyte subsets, in association with a stress response characterized by increased plasma epinephrine levels; (2) hypoglycemia increased platelet reactivity, promoted formation of MPAs and promoted aggregate formation between proinflammatory monocytes and platelets; (3) leucocyte mobilization to the stress response of low-dose endotoxin was independent of epinephrine, and antecedent hypoglycemia resulted in a significantly higher inflammatory leucocyte response to low-dose endotoxin administered 48 hours later.

As shown previously (13, 37), we confirm that hypoglycemia results in leucocytosis. In addition, we present, for the first time, the effect of hypoglycemia on monocyte subset kinetics and demonstrate an increase in the absolute number of all three circulating monocyte subsets. The largest increase was observed in numbers of circulating NCM (2-fold) and IM

(1.8-fold) with a modest increase in the number of CM (1.3-fold). These data are in keeping with an observed selective mobilization of $CD16^+$ monocytes in response to exercise (27, 38) and epinephrine infusion (39). Ratter et al. also recently determined that hypoglycemia might modify selective monocyte mobilization (37). However, they did not phenotype monocyte subsets but rather measured total levels of CD16 on peripheral blood mononuclear cells isolated from both healthy participants and those with type 1 diabetes in experimental hypoglycemia settings. Our data identify specific changes in monocyte subsets that have been previously linked to monocyte activation and atherogenesis. As observational data support the notion of $CD16^+$ monocytes being proatherogenic (20, 22-24), and adrenergic modulation of monocytes induces proinflammatory changes (40), an increase in the circulating number of these cells following hypoglycemia may enhance CV risk in diabetes.

Previous studies investigating effects of hypoglycemia on platelet biology have suggested an increase in platelet reactivity; however, this was in the context of significant hypoglycemic stimulus as part of an insulin stress test (41). An older investigation into the effect of hypoglycemia on monocyte-platelet interactions in type 1 diabetes and healthy controls has also suggested a trend towards increased MPA formation, but these data were not conclusive with little difference between euglycemic and hypoglycemic conditions (42). Our study also recapitulates and extends previous findings that hypoglycemia is prothrombotic, as evidenced by an increased platelet count and increased platelet reactivity to ADP (43). We have now conclusively demonstrated an overall increase in formation of MPA in hypoglycemia in comparison to euglycemia. Furthermore, we provide novel data demonstrating MPA formation within monocyte subsets in experimental hypoglycemia. MPA formation is a highly sensitive marker of both monocyte and platelet activation (44, 45). MPA formation promotes monocyte release of the proinflammatory cytokines; TNFα, CXCL8 and CCL2 (46,

443 47) and increases adhesive properties of monocytes (48), thereby representing a bridge
444 between inflammation and thrombosis, that may serve to increase CV risk. In acute coronary
445 syndromes, MPA formation correlates with troponin elevation, risk of in-hospital cardiac
446 events including death and risk of future cardiac events (20, 49). We have also shown that
447 NCM and IM aggregate more readily with platelets in response to hypoglycemia compared to
448 CM. A similar observation of proportionally higher IM-MPA and NCM-MPA formation has
449 been reported in patients following an ST-elevation myocardial infarction (STEMI) with
450 higher IM-MPAs in particular being a poor prognostic indicator at 6 weeks following STEMI
451 (20). Thus, our data suggest that hypoglycemia not only increased circulating numbers of
452 $CD16^+$ monocytes, but also promoted increased interaction between these proinflammatory
453 monocyte subsets and platelets.
454
455 In a first model of its type, we wished to determine whether antecedent hypoglycemia
456 modulated responses to low-dose endotoxin. We chose a low-dose endotoxin model firstly
457 because we felt it the safest way to combine the clamp and endotoxin human models,
458 secondly because future extension to the study of people with diabetes would be more
459 feasible with this model, and finally because people with diabetes are often exposed to
460 chronic low-grade infections through foot ulceration and periodontitis which might further
461 increase the risk of CV mortality (50, 51). In our model, we observed in all groups that
462 monocytes were activated even in response to low-dose endotoxin, as indicated by
463 upregulation of systemic levels of the CX_3CR1 ligand CX_3CL1, and the upregulation of the
464 adhesion molecule CD11b on the monocytes themselves. Interestingly, compared to
465 euglycemia, hypoglycemia resulted in greater leucocyte mobilization in response to low-dose
466 intravenous endotoxemia 48 hours later. Furthermore, we noted a non-significant trend
467 towards a higher total monocyte count in the hypoglycemia group 4 hours post endotoxin

compared to euglycemia. The percentage of monocytes that were CD11b positive was also higher in hypoglycemia group compared to euglycemia group at 2 hours post endotoxin. Levels of leucocyte mobilization were similar between groups who received prior sham-saline or hypoglycemia. These data suggest that euglycemia with insulin suppressed leucocyte mobilization in response to endotoxin 48 hours later, consistent with the known antiinflammatory actions of insulin (52, 53) and that the physiological stress of hypoglycemia overcame this insulin-mediated suppression of inflammatory responses. Our data show that drivers for differential leucocyte mobilization to endotoxin are unlikely to be due to differences between groups in epinephrine, cortisol and growth hormone levels post endotoxin as these were not significantly different. Our observation that a single episode of hypoglycemia compared to euglycemia invokes a stronger proinflammatory response to endotoxin up to 2 days later is of potential clinical relevance given that trial data suggest downstream mortality following hypoglycemia (29-31).

The strengths of our study include use of a novel human experimental model and detailed flow cytometric analysis that allowed us to comprehensively describe immune cell kinetics and activation status in response to experimental hypoglycemia and endotoxin challenge *in vivo*. The separation of clamp and endotoxin studies by 48 hours allowed us to probe the longitudinal effects of hypoglycemia on innate immunity. Moreover, by using a sham-saline group, we specifically controlled for the immunological effects of insulin, thereby robustly investigating proinflammatory changes in response to hypoglycemia.

One limitation was our decision to study a relatively small number of young healthy participants. This limits the applicability of our findings to older patients with diabetes, established CV risk factors and atherosclerosis. For ethical and safety reasons, we decided to

examine our novel experimental model initially in healthy participants. We also specifically adopted a low-dose endotoxin model with future translatability in older, higher-risk participants in mind. Future studies should therefore confirm our findings in those with diabetes. In addition, it is worth noting that we studied cell numbers, phenotypic changes and activation status in circulating immune cells and this may not necessarily reflect the functional capacity of these cells in an atherosclerotic plaque. An animal model of combined experimental hypoglycemia and atherosclerosis may help to resolve these questions.

In conclusion, hypoglycemia mobilized proatherogenic monocyte subsets and induced prothrombotic changes by increasing platelet reactivity. In addition, hypoglycemia amplified interactions between platelets and monocytes by promoting MPA formation with enhanced aggregation of proinflammatory monocytes with platelets. Hypoglycemia may also prime the innate immune system to respond more robustly to stimuli such as endotoxin. This implies proinflammatory consequences of hypoglycemia beyond the acute episode. These data provide novel mechanistic insights into how hypoglycemia could increase CV risk through upregulation of inflammatory responses.

Acknowledgements

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Table & Figure legends

Table 1: Comparison of participant characteristics at baseline. Data are mean ± SD or median (interquartile range). *P* values indicate comparisons between study groups via parametric or nonparametric testing. BMI, body mass index; WBC, white blood cells.

Figure 1: Glucose, insulin and counter-regulatory hormones in clamp studies. Arterialized whole blood glucose values during hyperinsulinemic hypoglycemic, euglycemic and sham-saline clamps (a), epinephrine (b), free insulin (c), cortisol (d) and growth hormone values (e) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), *P<0.05, **P<0.01, ***P<0.001, ns-non-significant, P-values are provided for comparison between study groups. Black circles (and dashed line 1a)-hypoglycemia group; open circles-euglycemia group; black triangles (and solid line 1a)-sham-saline group.

Figure 2: Peripheral total white blood cell, neutrophil and lymphocyte kinetics in experimental hypoglycemia and controls. Number of circulating total WBCs (a), neutrophils (b) and lymphocytes (c) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), **P<0.01, ***P<0.001, P-values are provided for comparison between study groups. Black circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group. WBC, white blood cells.

Figure 3: Total monocyte count and monocyte subset kinetics in experimental hypoglycemia and controls. Absolute circulating numbers of total monocytes (a) and monocyte subsets comprising of NCM (b), IM (c) and CM (d) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), *P<0.05, **P<0.01, ***P<0.001, P-values are provided for comparison between study groups. Black

720 circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline
721 group. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical
722 monocytes.
723
724 **Figure 4: Platelet reactivity and monocyte-platelet aggregate formation in experimental**
725 **hypoglycemia and controls.** Total platelet count (a), platelet aggregation to ADP 6.45 μM
726 (b), total MPA formation (c) and MPA formation within monocyte subsets; NCM-MPA (d),
727 IM-MPA (e) and CM-MPA (f) after 60 minutes of hypoglycemia, euglycemia or sham-saline
728 injection. Data are mean (SEM), *P<0.05, **P<0.01, ns-non-significant, P-values are
729 provided for comparison between study groups. Black circles-hypoglycemia group; open
730 circles-euglycemia group; black triangles-sham-saline group. MPA, monocyte-platelet
731 aggregates; NCM-MPA, non-classical monocyte-platelet aggregates; IM-MPA, intermediate
732 monocyte-platelet aggregates; CM-MPA, classical monocyte-platelet aggregates.
733
734 **Figure 5: Changes in epinephrine, cortisol and growth hormone response post**
735 **endotoxin challenge.** Epinephrine (a), cortisol (b) and growth hormone (c) responses 2, 4
736 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants
737 that underwent hypoglycemia, euglycemia or a sham-saline clamp 48 hours earlier. Data are
738 mean (SEM), **P<0.01, ns-non-significant, P-value on dashed line in (b) represents change
739 in cortisol at 4 hours compared to baseline in all groups, solid horizontal lines represent
740 significance for comparison between study groups. Dashed line in (a) illustrates the mean
741 epinephrine response in hypoglycemia clamp subjects. Black circles-hypoglycemia group;
742 open circles-euglycemia group; black triangles-sham-saline group.
743

Figure 6: Peripheral total white blood cell, neutrophil and lymphocyte kinetics post endotoxin challenge. Number of circulating total WBCs (a), neutrophils (b) and lymphocytes (c) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycemia, euglycemia or a sham-saline clamp 48 hours earlier. Data are mean (SEM), *P<0.05, ns-non-significant, P-values are provided for comparison between study groups, solid horizontal line in (c) represents significance for comparison between study groups. Black circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group.

Figure 7: Total monocyte count and monocyte subset kinetics post endotoxin challenge. Absolute circulating numbers of total monocytes (a) and monocyte subsets comprising of NCM (b), IM (c) and CM (d) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycemia, euglycemia or a sham-saline clamp 48 hours earlier. Data are mean (SEM), ***P<0.001, ns-non-significant, P-value on dashed line in (b) represents change in number of NCMs at 2, 4 and 6 hours compared to baseline in all study groups. P-value on dashed line in (c) represents change in number of IM at 2, 4 and 6 hours compared to baseline in all study groups. P-value on dashed lines in (d) represent change in number of CM at 2 and 6 hours compared to baseline in all study groups. Solid horizontal lines represent significance for comparison between study groups. Black circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical monocytes.

Parameter	Hypoglycemia	Euglycemia	Sham-saline	*p*
Total *n=24*	8	8	8	N/A
Sex (M/F)	4/4	4/4	4/4	N/A
Age (years)	21 (19-22)	21 (20-23)	21.5 (21-26)	0.299
BMI (kg/m^2)	24 ± 2	23 ± 2	24 ± 4	0.638
HbA_{1c} % mmol/mol	 5.2 ± 0.31 34 ± 3.6	 5.2 ± 0.26 33.5 ± 2.8	 5.1 ± 0.14 32.6 ± 1.4	 0.792 0.616
Total WBC ($x10^9/l$)	6.26 ± 1.42	4.83 ± 0.91	4.50 ± 1.69	0.102

Figure

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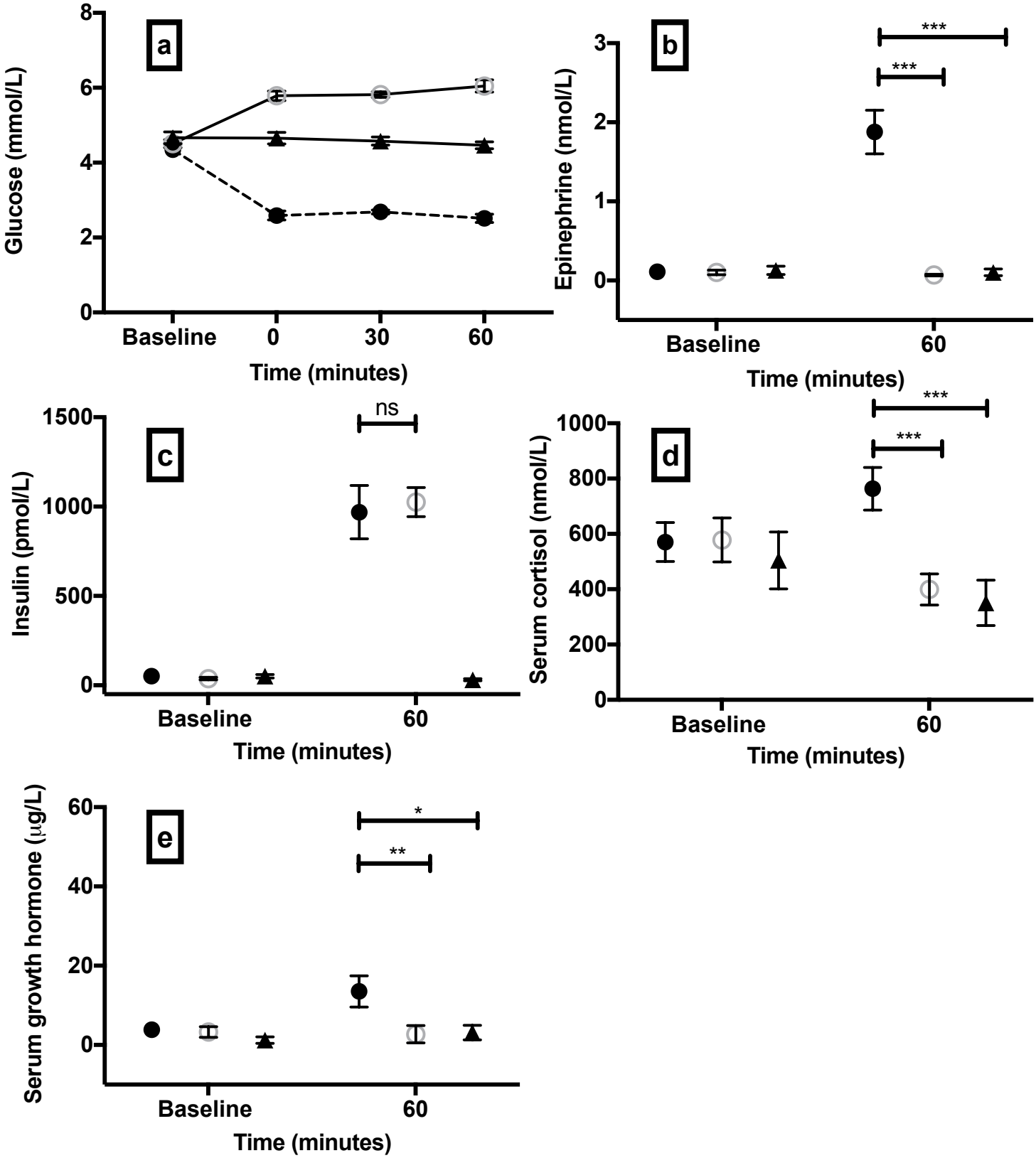

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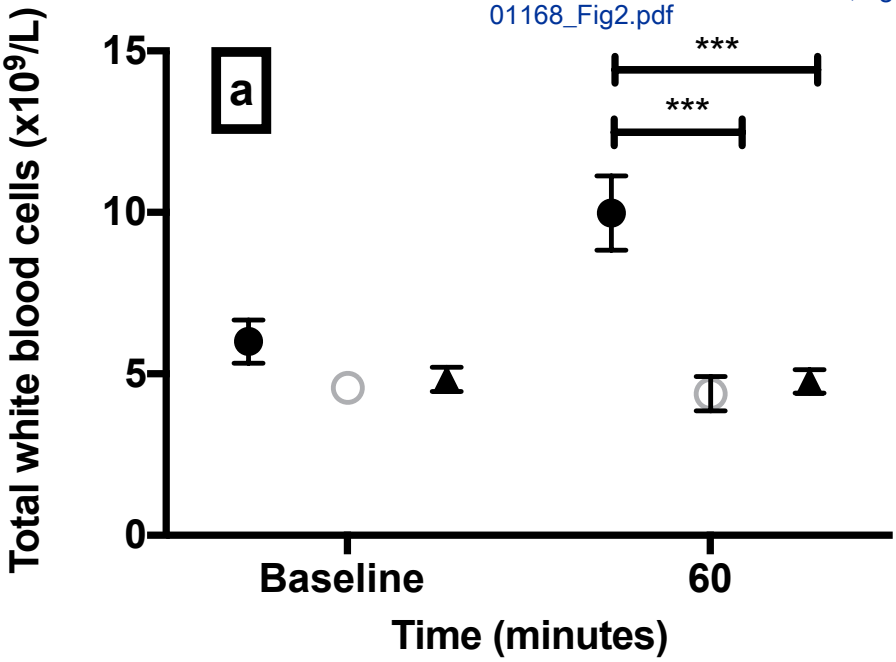

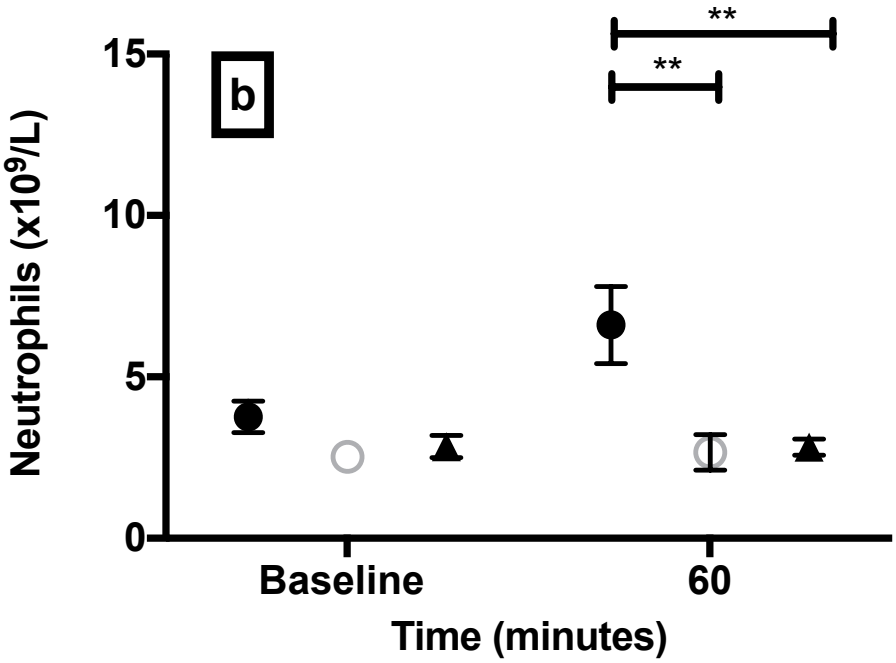

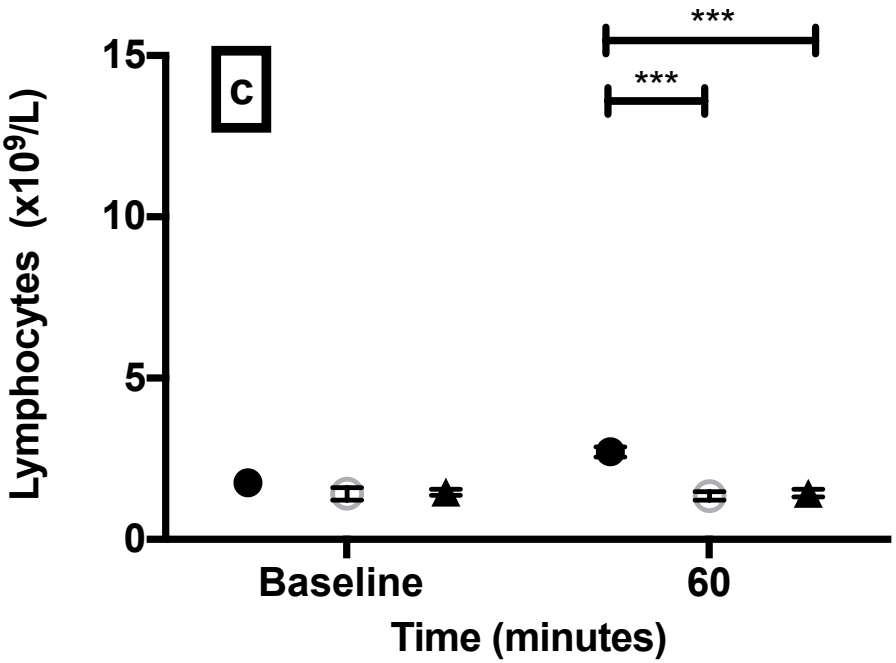

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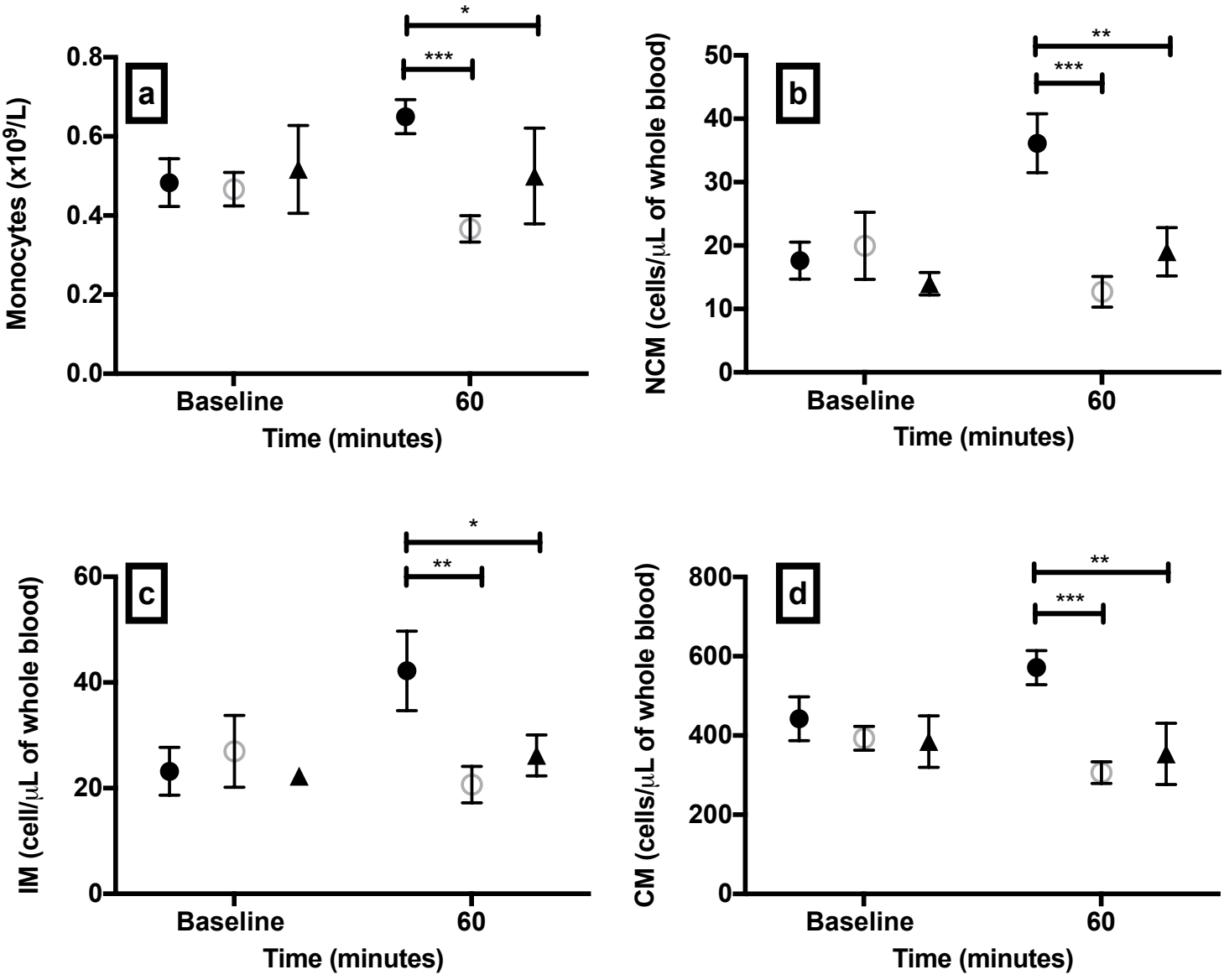

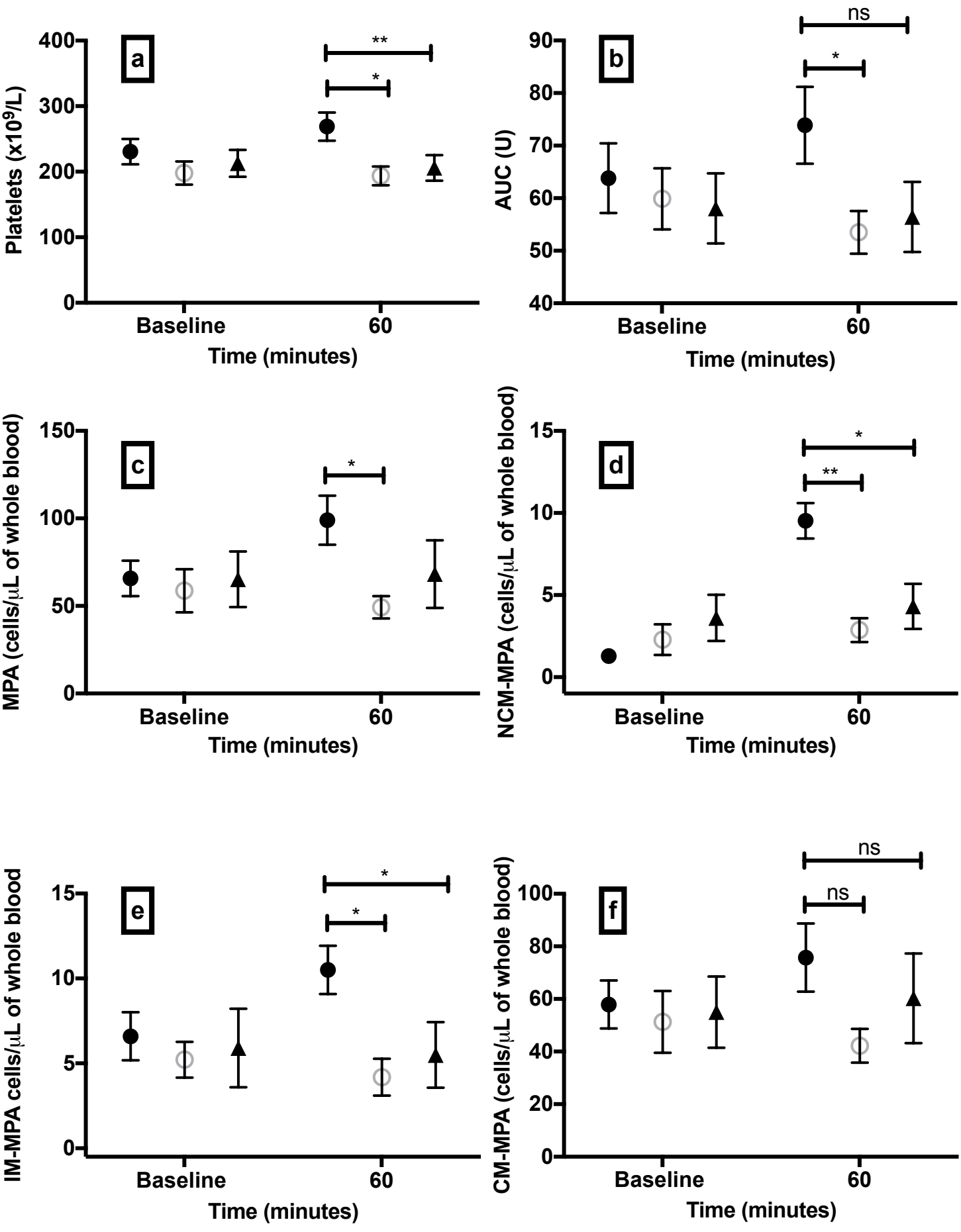

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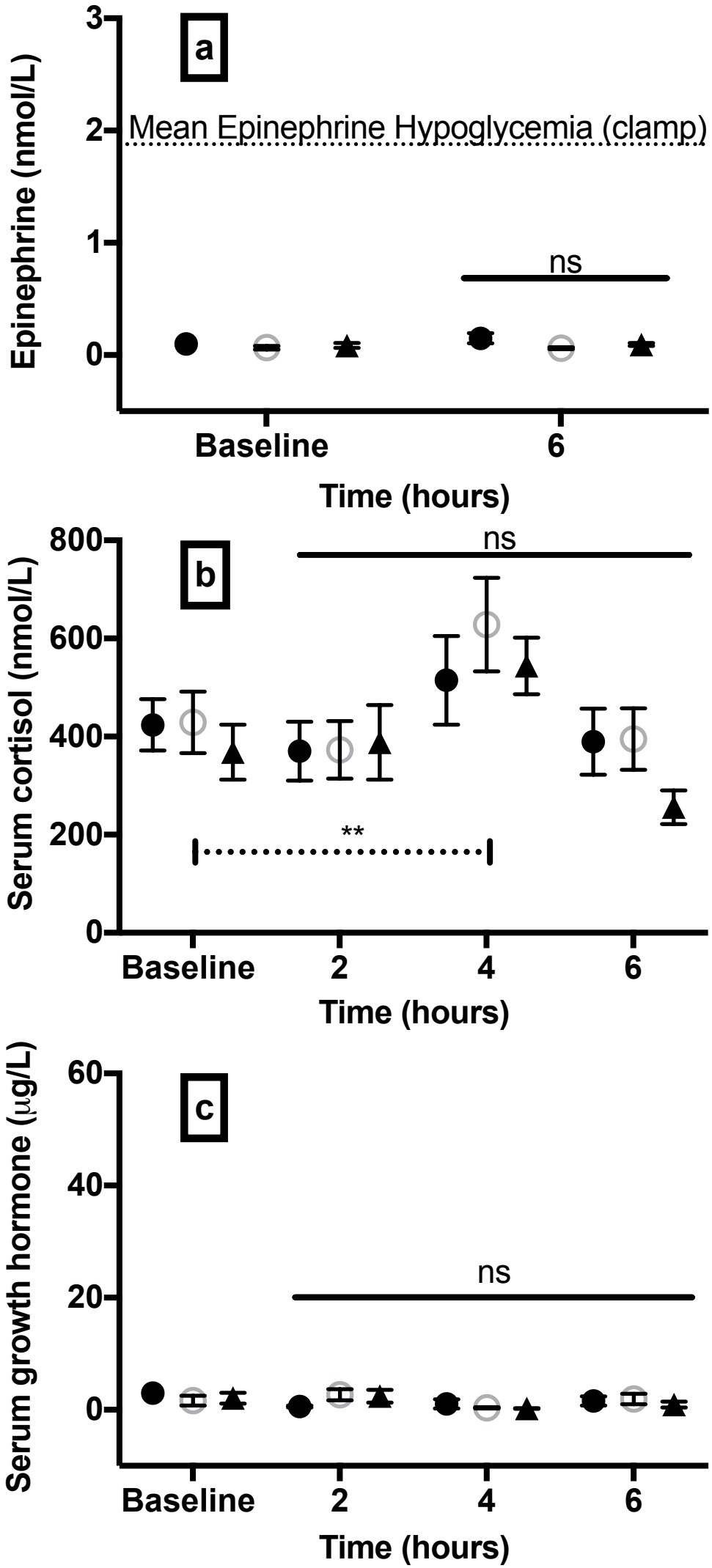

Figure

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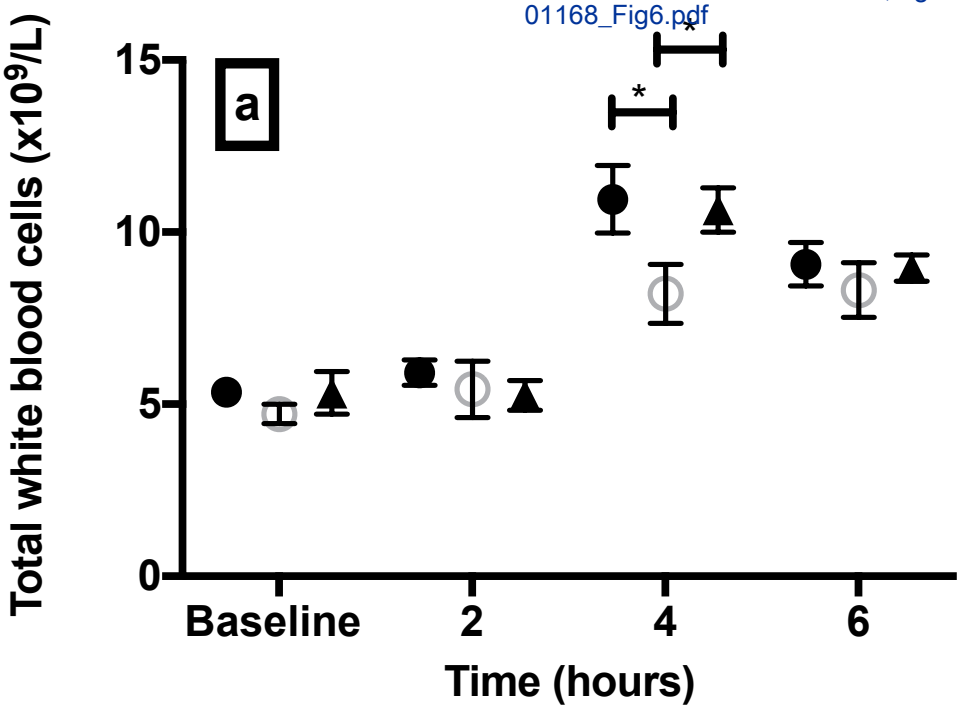

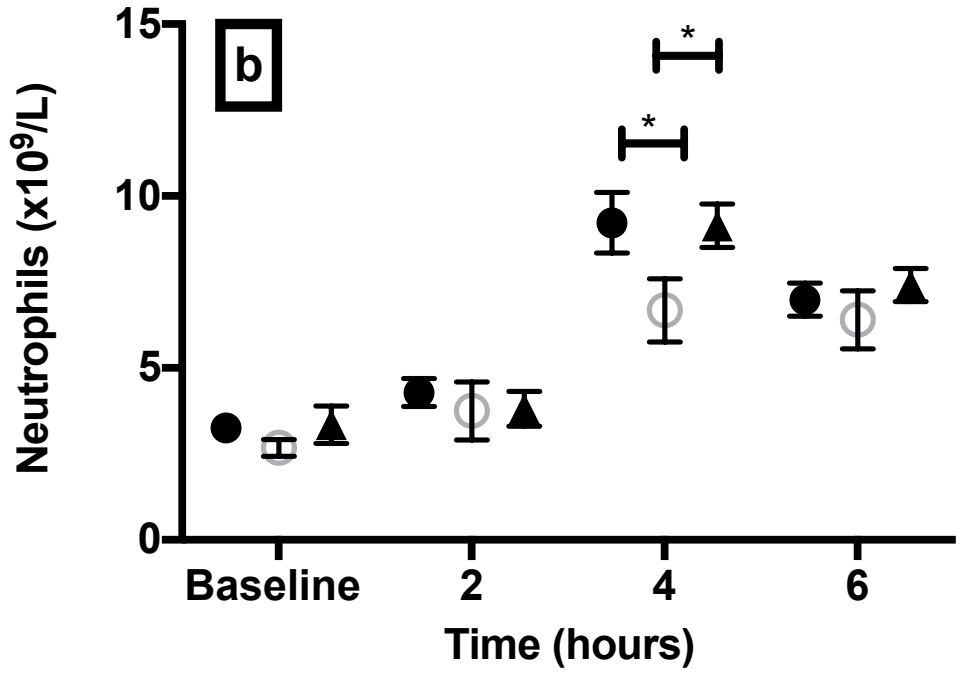

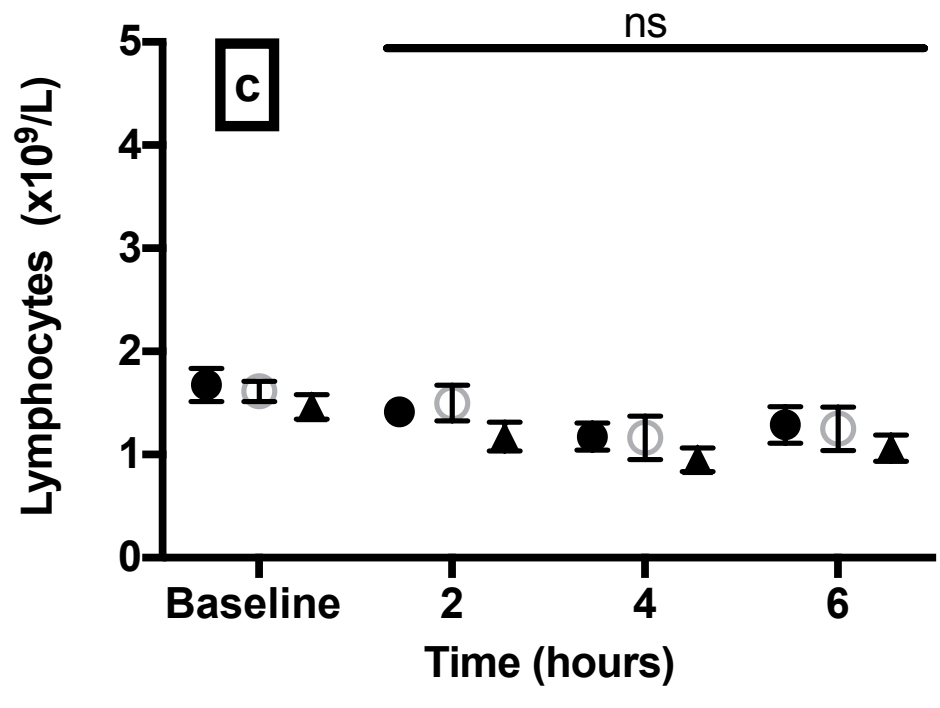

Figure

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